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Introduction and Theory

In light-scattering studies carried out on salt-free isoionic solutions of proteins, dilution with distilled water displaces the pH of the protein solution toward neutral, the theoretical pH at infinite dilution being 7.0. For reasons of electrical neutrality, the protein molecules become progressively ionized with dilution. If, however, the diluting solvent is an acid or alkaline solution of the proper concentration to give a pH equal to the isoionic point of the protein, the pH remains close to isoionic over the normal concentration range with the result that the ionization is greatly suppressed.

The progressive ionization of the protein results in a change of the derivative of the excess chemical potential of the protein with respect to its concentration. From considerations of electroneutrality, it is possible to calculate the magnitude of this effect using expressions developed by Kirkwood (1).

For the case of a protein in water, electroneutrality can be expressed by:

$$\sum_{\alpha} Z_{\alpha} m_{\alpha} + [H^{+}] - [OH^{-}] = 0 \qquad (1)$$

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where Z_α is the charge on a protein molecule of degree of ionization α, and m_α is its concentration in moles per liter.

If the diluting agent is an acid, this relation becomes:

$$\sum_\alpha Z_\alpha m_\alpha + [H^+] - [OH^-] - [A^-] = 0 \qquad (1a)$$

where $[A^-]$ is the concentration of the acid anion.

The protein molecule may for simplicity be considered as an ampholyte containing 2ν basic sites, namely, ν negatively charged sites and ν neutral sites, for example —COO^- and —NH_2 (although this includes all ionizable groups), to which protons can be attached. Then, the protein molecule is in the neutral state when ν protons are bound to it.

We can express now the activity coefficient of the protein, γ, as the product of its activity coefficient in the neutral state, γ_ν, and the fraction of protein in the neutral state at any given concentration, f_ν. The derivative of the excess chemical potential with respect to protein concentration then is:

$$\frac{1}{RT}\frac{\partial \mu_2^{(e)}}{\partial m_2} = \frac{\partial \log \gamma_\nu}{\partial m_2} + \frac{\partial \log f_\nu}{\partial m_2} \qquad (2)$$

The first term on the right-hand side of this equation represents the departure from ideal behavior of the neutral protein and is given by (2):

$$\frac{\partial \log \gamma_\nu}{\partial m_2} = -\frac{\pi N e^4 \langle Z^2 \rangle_{Av}^2}{(DkT)^2 \kappa (1 + \kappa a)^2} + \frac{7}{6}\pi N a^3 + 2B' \qquad (3)$$

where $<Z^2>_{Av}$ is the mean square charge of the protein molecule in protonic units e, N is Avogadro's number, k is Boltzmann's constant, T is the thermodynamic temperature, D is the dielectric constant of the solvent, and κ and α are the Debye-Hückel parameters. The second term of this equation is the excluded volume, and B' reflects the combined effect of all types of intermolecular force.

From Eq. (40) of Ref. (1) we have:

$$f_\nu = \frac{K_\nu [H^+]^\nu}{G(H^+)}$$

$$G(H^+) = \sum_{k=0}^{2\nu} K_k [H^+]^k \qquad (4)$$

$$K_n = \gamma_n^{-1} \prod_{s=1}^{n} K_{2\nu-s+1}^{-1}$$

where $K_{2\nu-s+1}$ is the dissociation constant of the s'th group, and γ_n is the activity coefficient of the ion H_nP, assumed to be independent of pH as in the treatment of Linderstrøm-Lang (3). Differentiation of the first of Eq. (4) with respect to protein concentration yields for the second terms on the right-hand side of Eq. (2):

$$\frac{d \log f_\nu}{dm_2} = \left(\frac{\nu}{[H^+]} - \frac{d \log G}{d[H^+]} \right) \frac{d[H^+]}{dm_2} \tag{5}$$

Differentiating Eq. (1) or (1a) with respect to protein concentration, we obtain:

$$\frac{d[H^+]}{dm_2} = \frac{-\bar{Z}}{1 + \frac{K_w}{[H^+]^2} + m_2 \frac{d\bar{Z}}{d[H^+]}} \tag{6}$$

where $\bar{Z}$ is the mean charge of the protein.

From Eq. (41) of Ref. (1), we have:

$$\frac{d \log G}{d[H^+]} = \frac{\bar{Z} + \nu}{[H^+]} \tag{7}$$

Combining Eqs. (5), (6), and (7), we obtain finally:

$$\frac{d \log f_\nu}{dm_2} = \frac{\bar{Z}^2}{[H^+]} \frac{1}{1 + \frac{K_w}{[H^+]^2} + m_2 \frac{d\bar{Z}}{d[H^+]}} \tag{8}$$

For the limiting case of extrapolation to zero protein concentration and a pH of 7.0, this reduces to:

$$\frac{d \log f_\nu}{dm_2} = \frac{\bar{Z}_0^2}{2[H^+]} \tag{9}$$

Substituting Eqs. (2) and (8) into the equation of light scattering for a two-component system (4), we obtain:

$$H \frac{C_2}{\tau} = \frac{1}{M_2} \left[1 + \frac{C_2}{M_2} \left(\frac{d \log \gamma_\nu}{dm_2} + \frac{\bar{Z}^2}{[H^+]} \frac{1}{1 + \frac{K_w}{[H^+]^2} + m_2 \frac{d\bar{Z}}{d[H^+]}} \right) \right] \tag{10}$$

where C_2 is the protein concentration in grams per liter, and M_2 is its molecular weight.

By solving simultaneously Eq. (1) or (1a) with the titration curve of a protein, it is possible to evaluate the contribution to light scattering of the term represented by Eq. (8). Such calculations have been carried out

for bovine serum albumin (BSA) and conalbumin, using titration data of Tanford *et al.* (5) and of Lowey (6), respectively. The values calculated for the contribution of the ionization term to light scattering as well as experimentally measured values of $\frac{HC_2}{\tau}$ for these two proteins (7, 8, 9) in salt-free isoionic solution and for BSA in 1×10^{-5} *M* HCl are summarized in Table I.

In the case of dilution with water it can be seen that, while for conalbumin, which has its isoionic point at pH 6.75, the contribution of the term resulting from progressive ionization is negligibly small even at the lowest concentration measured, this effect assumes a large magnitude already at a protein concentration of 0.05% for serum albumin, and its value becomes larger than the intercept at 0.005%.

That no positive term of comparable magnitude is observed experi-

TABLE I

Effect of Ionization of Protein on Light Scattering

	in water				in 1×10^{-5} *M* HCl		
Prot. conc. g./l.	$\left(H\frac{C_2}{\tau}\right)_{obs.} \times 10^5$	$[H^+] \times 10^6$	$\bar{Z}$	$\left[\frac{C_2}{M_2^2}\frac{d\log f_\nu}{dm_2}\right]$	$[H^+] \times 10^5$	$\bar{Z}$	$\left[\frac{C_2}{M_2^2}\frac{d\log f_\nu}{dm_2}\right]$
			Bovine Serum Albumin				
6.0	1.047	—	—	—	1.26	−0.03	0.3×10^{-8}
3.0	1.115	—	—	—	1.25	−0.06	1.4×10^{-8}
1.4	1.165	10.00	−0.50	7.06×10^{-7}	—	—	—
1.0	1.181	—	—	—	1.22	−0.15	7.1×10^{-8}
0.5	1.207	7.94	−1.14	3.33×10^{-6}	1.19	−0.26	1.77×10^{-7}
0.3	1.220	6.31	−1.57	5.99×10^{-6}	1.16	−0.35	2.69×10^{-7}
0.2	1.230	5.00	−2.14	1.024×10^{-5}	1.13	−0.44	3.50×10^{-7}
0.1	1.241	3.98	−2.57	1.314×10^{-5}	1.09	−0.59	4.40×10^{-7}
0.08	1.245	3.16	−2.86	1.580×10^{-5}	1.06	−0.65	4.17×10^{-7}
0.05	1.248	2.51	−3.29	2.000×10^{-5}	1.05	−0.70	3.80×10^{-7}
0.02[a]	1.255	1.26	−4.57	3.397×10^{-5}	1.03	−0.80	2.28×10^{-7}
0.01[a]	1.259	0.79	−5.29	4.02×10^{-5}	1.01	−0.84	1.36×10^{-7}
0[a]	1.267	0.10	−12.0		1.00	−0.87	
			Conalbumin				
0.17	1.32	0.17	−0.05	1.3×10^{-8}			
0.06	1.34	0.16	−0.13	8.2×10^{-8}			
0.01[a]	1.35	0.14	−0.47	6.4×10^{-7}			
0[a]	1.36	0.10	−1.31				

[a] Outside of experimental range; $\left(H\frac{C_2}{\tau}\right)_{obs.}$ obtained from extrapolation of data at higher concentrations.

mentally is obvious from the data presented in col. 2 of Table I. It must be kept in mind that the above calculation is based on the assumption that no ions other than protein, hydrogen, and hydroxyl are present in the system at any dilution, and that extrapolation to zero protein concentration constitutes a true extrapolation to pH 7.0. That this ideal situation is not attainable in the presently available experimental environment should be evident. Furthermore, the conductivity data both on the deionized protein and the distilled water used as diluting solvent do not permit drawing any conclusions beyond the fact that the ionic strength is no greater than 1×10^{-5}. Indeed, the fact that light-scattering measurements in deionized "salt-free" solution and in 1×10^{-5} *M* NaCl fall on the same curve (9) indicates that the ionic strength of the "salt-free" protein solution due to small ions is of the order of 1×10^{-5}. A more desirable approach would be a check of this theory using an isoionic protein solution in a known constant low concentration of acid. When the light-scattering measurements were carried out in 1×10^{-5} *M* HCl (pH = 5.0, which corresponds to the isoionic point of the protein, pH = 4.9), it was found that the light-scattering data agreed well (7, 9) with that obtained in the "salt-free" state.

For the case of 1×10^{-5} *M* HCl, the relation between the mean charge of the protein and the hydrogen-ion concentration is:

$$\bar{Z} = \frac{1}{m_2}\left([\mathrm{Cl^-}] + \frac{K_w}{[\mathrm{H^+}]} - [\mathrm{H^+}]\right) \tag{11}$$

When this is solved simultaneously with the titration curve, and the values obtained for a series of protein concentrations are substituted into Eq. (7), the values shown in col. 8 of Table I are obtained. It can be seen that, in this case, the ionization effect has been greatly suppressed, and the contribution to light scattering of progressive ionization has the property of passing through a maximum at a low concentration. For BSA in 1×10^{-5} *M* HCl, this contribution is never greater than 3.5%. This is the value obtained at a protein concentration of 0.01% which is the maximum point. If this term is subtracted from our experimental curve at the lowest concentrations measured and the resulting curve is extrapolated to zero concentration, we find that the value of the intercept comes into better agreement with the intercept obtained in 1×10^{-3} *M* NaCl (7). If to the extrapolated curve we then add the calculated value of the ionization term, the light-scattering plot first rises with concentration, passes through a maximum at 0.01%, and drops

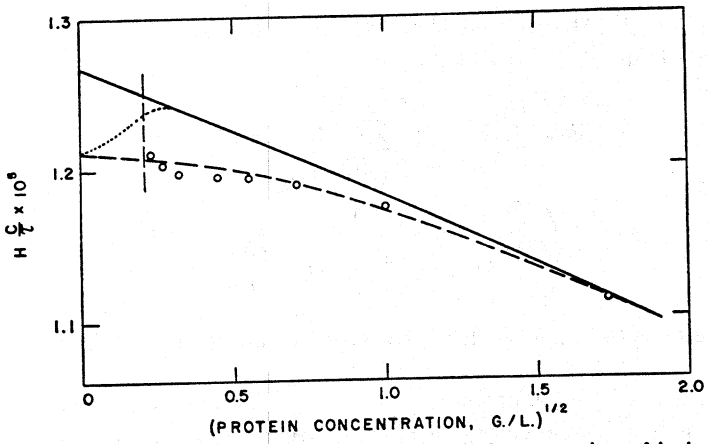

FIG. 1. Effect of progressive ionization on the light scattering of isoionic Armour bovine serum albumin in 1×10^{-5} M HCl (region below 0.3% protein).

Solid line (———): least-squares curve of the experimental points; O = experimental points from which the ionization term had been subtracted. Dashed line (- - - -): curve fitting best the corrected points (extrapolated to zero protein concentration). Dotted line (····): curve obtained by adding calculated values of ionization term to the dashed line. (The vertical dashed line represents the lower limit of experimental data, 0.005%).

again as shown by our data. These effects are demonstrated in Fig. 1 in which the experimental data below 0.3% protein and the calculated corrections are plotted as a function of $C_2^{1/2}$ for BSA in 1×10^{-5} M HCl.

Since the curvature observed in the light-scattering work with isoionic salt-free proteins (7, 8, 9) has been attributed to the presence of an attractive force due to charge fluctuations on the protein molecules (2), it is of interest to determine the effect of the introduction of the ionization term on the interpretation of the data. Examination of the data on conalbumin in distilled water shows that the change in the curvature of the light-scattering plot resulting from correction for the ionization term is negligibly small. In the case of BSA in 1×10^{-5} M HCl, however, the presence of the added electrolyte has to be taken into account in the calculation of the fluctuating charge of the protein from the light-scattering data. In this case, the simple square root relation (7, 8, 9) is no longer valid at low concentrations of protein, since the contribution of the protein and of the added acid to ionic strength, and therefore to the Debye-Hückel κ, are of similar magnitude. Taking into account both the ionization term [Eq. (8)] and the contribution of the added electro-

lyte, after binominal expansion of $(1 + \kappa\alpha)^{-2}$ in Eq. (3), the light-scattering equation [Eq. (10)] becomes:

$$H\frac{C_2}{\tau} = \frac{1}{M_2}\left[1 - \frac{\pi^{1/2}N^{1/2}e^3}{2(DkT)^{3/2}M_2^{1/2}} \frac{\langle Z^2\rangle_{Av}^{3/2}C_2^{1/2}}{\left(1 + \frac{2M_2 m_{HCl}}{\langle Z^2\rangle_{Av}C_2}\right)^{1/2}} + \frac{C_2}{M_2}\left(\frac{\bar{Z}^2}{[H^+]} \frac{1}{1 + \frac{K_w}{[H^+]^2} + m_2\frac{d\bar{Z}}{d[H^+]}} + 2B\right)\right] \tag{12}$$

where B represents the sum of all other contributions to the coefficient of C_2.

An examination of this equation reveals that the two effects act in opposite directions and tend to balance out. Correction for the ionization term results in a lowering of the light-scattering curve, as shown in Fig. 1, while the contribution of the added electrolyte to the ionic strength raises the points by a comparable amount. A calculation of the value of $<Z^2>_{Av}^{1/2}$ for isoionic BSA using Eq. (12) was carried by the method of successive approximations on previously reported data (7). This resulted in a value of 3.5 protonic units for the magnitude of the fluctuating charge of this protein, which is identical with the value previously calculated (7) by means of the uncorrected simple square-root equation for the light scattering of isoionic salt-free proteins. Thus, in the cases of conalbumin in distilled water and BSA in 1×10^{-5} M HCl, progressive ionization of the protein with dilution does not lead to significant revisions of the value of the fluctuating charge calculated from the simpler equation (7).

Summary

The effect on light scattering of progressive ionization with dilution of isoionic proteins has been examined. An equation is developed for the calculation of this effect. It is found that, in the case of bovine serum albumin, this can take on a very large magnitude at low concentration if dilution is carried out with distilled water. If the protein is kept close to its isoionic pH by using 1×10^{-5} M HCl as diluting agent for BSA, or if the isoionic point is close to pH 7.0, as is the case with conalbumin, this effect is greatly suppressed and causes the light-scattering plot to go through a maximum point at very high dilution.

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